PATENT
Docket No.: 176/60792 (6-11415-868)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Mahin D. Maines	Examiner: D. Ramirez
Serial No. :	09/606,129	
Cnfrm. No. :	5529	
Filed :	June 28, 2000	Art Unit: 1652
For :	BILIVERDIN REDUCTASE FRAGMENTS AND VARIANTS, AND METHODS OF USING BILIVERDIN REDUCTASE AND SUCH FRAGMENTS AND VARIANTS	

THIRD DECLARATION OF MAHIN D. MAINES UNDER 37 C.F.R. § 1.132

1. I am the inventor of the above-identified application.

2. I am currently Professor of Biochemistry and Biophysics at the University of Rochester Medical Center, Rochester, NY.

3. I received a B.A. in Biology from Ball State University in 1964, an M.S. in Chemistry from Ball State University in 1967, and a Ph.D. in Pharmacology from the University of Missouri in 1970. A major focus of my career has been involved with biliverdin reductase, its activities, its properties, and how the protein interacts with other cellular components to regulate cell activities. Since 1981, I have published over twenty-four articles concerning different aspects of biliverdin reductase.

4. I am presenting this declaration to demonstrate that the *in vitro* results obtained using biliverdin reductase ("BVR") and fragments thereof for modifying protein kinase C ("PKC") activity, described in Example 3 of the above-identified application, are predictive of results that can be achieved *in vivo*.

5. To demonstrate *in vivo* efficacy of BVR, I transfected 293 cells with either (1) an empty vector (pCDNA3), (2) a vector capable of inducing human BVR expression (pCDNA3-BVR), or (3) a retroviral construct containing small interference

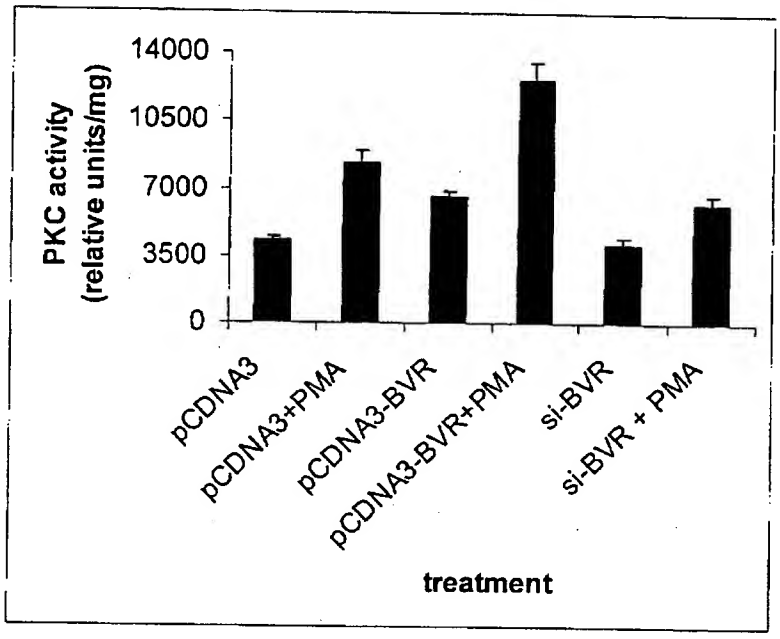
fragments for BVR (si-BVR). The transfected cells were starved over night with growth media containing 0.1% FBS. Cells were than treated with 100 nM phorbol-12-myristate-13-acetate ("PMA") for 20 min and lysed with buffer containing: 20 mM MOPS, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM Na Vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP-40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF), 10 μg/ml leupeptin and aprotinin. Cell lysates were sonicated, centrifuged 15 min at 13 krpm, and supernatants were processed for determination of protein content by using Bradford assay and for determination of PKC activity by using ELISA kit from Stressgen.

6. The results of the Bradford assay allowed for quantitation of proteins present in the lysates sample obtained from the transfected cells. This allowed for normalizing the ELISA results discussed below.

7. The results of the ELISA assay are presented in the table below and graphically in the figure below.

Vector	PKC activity (rel. units/mg)	Percent Change (rel. control)
pCDNA3 (control)	4284	
pCDNA3+PMA	8372	95
pCDNA3-BVR	6622	55
pCDNA3-BVR+PMA	12600	194
si-BVR	4138	(3)
si-BVR + PMA	6210	45

In the table above, PKC activity is expressed as an average OD normalized to the amount of the proteins present in the sample.



PMA is a known activator of protein kinase C, both *in vivo* and *in vitro*. As expected, the introduction of PMA showed an increase in PKC activity for each transfection (i.e., comparing each transfection with and without PMA). The effect of BVR on *in vivo* PKC activity is clearly demonstrated, because the BVR-transfected cells displayed a substantial increase in PKC both with and without PMA.

8. Given the consistency between the *in vitro* results reported in Example 3 of the present application and the *in vivo* results described above, persons of skill in the art would expect that other *in vitro* results described in Example 3 would likewise predict the same or similar outcomes for *in vivo* modification of PKC activity.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 1/13/05

Mahin Maines

Mahin D. Maines